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Gamma Interferon-Induced Expression of Class II MHC Antigens by Human Keratinocytes: Effects of Conditions of Culture

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Abbreviations

sodium azide	AZ
bovine pituitary extract	BPE
Dulbecco's modified Eagle's medium	DMEM
ethylene diamine tetraacetate	EDTA
fluorescence activated cell sorter	FACS
fetal calf serum	FCS
interleukin-2	IL-2
keratinocyte defined medium	KDM
keratinocyte growth medium	KGM
monoclonal antibodies	mAbs
mycosis fungoides	MF
major histocompatibility complex	MHC
phosphate buffered saline	PBS
recombinant gamma interferon	rIFN-
fluorescein isothiocyanate conjugated rabbit anti-mouse IgG	R/M-FITC
squamous cell carcinoma cell line	SCL-1

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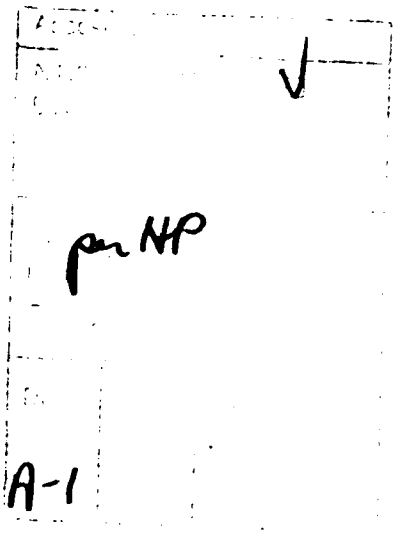

Abstract

Normal human keratinocytes grown in MCDB 153 plus bovine pituitary extract and treated with recombinant gamma interferon (rIFN-γ) express HLA-DR, DP and DQ antigens. The expression of these class II MHC antigens is time dependent: DR and DP appear before DQ. The delay in HLA-DQ expression is not due to the effects of trypsinization of cultures prior to analysis. Increasing the calcium ion concentration from 0.1 to 1.8 mM does not alter the expression of these antigens. Keratinocytes grown without serum proteins or bovine pituitary extract exhibited markedly delayed expression of DR. By contrast, keratinocytes grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum express DR and DP but only very small amounts of DQ after treatment with rIFN-γ. Expression of HLA-DR occurs at doses of rIFN-γ that are too low to cause growth inhibition. The cells of the squamous cell carcinoma cell line SCL-1, whether grown in MCDB 153 plus bovine pituitary extract or DMEM plus 10% fetal calf serum, express HLA-DQ and DP on only small numbers of cells after treatment with the lymphokine. Thus, the conditions of culture, possibly the presence of a serum factor(s), influence the expression of class II antigens in normal keratinocytes. Furthermore, rIFN-γ does not induce DP and DQ antigens readily in transformed squamous cells cultured in either serum-containing or serum-free medium.

Introduction

In a number of skin diseases including lichen planus, contact dermatitis, and mycosis fungoides (MF), including the poikilodermatous form of this disease, keratinocytes express class II antigens of the major histocompatibility complex (MHC) (1,2,3). In MF in particular, not only HLA-DR but also HLA-DQ and HLA-DP antigens are expressed by keratinocytes in lesional skin (4). We and others have reported recently that recombinant gamma interferon (rIFN-γ) induces the expression of HLA-DR antigen on human keratinocytes but little if any HLA-DQ (5,6). With the demonstration of DQ antigen expression on diseased skin in situ, we asked whether this expression could also be due to the effects of local secretion of IFN-γ by T cells and whether this expression was time dependent. Moreover, since another class II antigen of the MHC, named HLA-DP, has recently been described, we ascertained whether this antigen also could be induced by rIFN-γ (7).

Changes in the calcium ion concentration cause alterations in the growth pattern of human as well as murine keratinocytes; therefore, we determined whether changing the calcium ion concentration in human keratinocyte cultures resulted in changes of class II MHC antigen expression after rIFN-γ treatment (8,9). Also, we determined the effects of the presence and absence of serum on this induction.

The rIFN-γ not only induces expression of DR antigen, but also inhibits the growth of keratinocytes. In order to determine whether DR expression would occur without significant reduction in cell numbers, we performed a concentration curve comparing the effects of this lymphokine on DR expression and cell number in one and the same set of culture plates.

The rIFN-γ induces DR antigen on SCL-1 cells, a transformed human epithelial cell line (10). Since the regulation of class II antigens in neoplastic epithelial cells may be different than in normal keratinocytes, we also examined the induction of HLA-DQ and DP by rIFN-γ in SCL-1 cells under various conditions of culture.

Materials and Methods

Lymphokine, Monoclonal Antibodies (mAbs) and Cell Staining

The monoclonal antibodies anti HLA-DR, DP, and DQ were obtained from Becton Dickinson, Mountain View, CA. The isotype control mAbs, anti-Leu-2b and anti-Leu 3a were also purchased from Becton Dickinson. The mAbs, VM-1 and VM-2, directed against the basal cell layer of normal human skin, were developed in this laboratory and have been described previously (11,12). The rIFN-γ was a generous gift of Genentech Corp., South San Francisco, CA, and was used at 100 U/ml unless otherwise stated.

One million epidermal cells were stained for 30 min with the mAb diluted in 5% heat inactivated, fetal calf serum (FCS) in phosphate buffered saline (PBS) containing 0.02% sodium azide (AZ) as described previously (13). The cells were washed with 5% FCS/PBS/AZ, stained with fluorescein isothiocyanate conjugated rabbit anti-mouse IgG (R/M-FITC) (ICN Immunobiologicals, Lisle, IL) for 30 min, washed with and then resuspended in 5% FCS/PBS/AZ. The number of fluorescent cells was determined by fluorescence microscopy or fluorescence activated cell sorter (FACS) analysis (13). On the FACS, the mean fluorescence/cell was determined using a linear scale.

Cell Culture Conditions

Skin obtained from meloplasty specimens was processed as described previously (14). Single cell suspensions, consisting mainly of keratinocytes, were prepared as described (14). Other keratinocyte cultures, derived from breast skin, were obtained from Clonetics (San Diego, CA). Keratinocytes were grown using 3 different media:

complete medium, keratinocyte growth medium (KGM), and keratinocyte defined medium (KDM). In the first, dispersed cells were suspended in complete medium, which consists of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FCS, 50 ug/ml gentamicin and 2 mM L-glutamine, and were seeded at 1.8-2.2 x 10^6 cells/3.5 cm collagen-coated petri dish (Lux, Miles Scientific, Naperville, IL) (14). (The calcium ion concentration for DMEM is 1.8 mM.) For culture in serum free medium, the method described by Ham & Boyce was used (9,15). The cells were trypsinized and seeded at 3 x 10^4 cells/3.5 cm Petri dish in KGM (Clonetics), which contains bovine pituitary extract (BPE). The unadjusted calcium ion concentration in KGM is 0.1 mM. For some experiments, we increased the calcium ion concentration to 1.8 mM by adding appropriate amounts of $CaCl_2$ (high calcium medium). Using the third method, adult keratinocytes were grown in KDM (Clonetics), which is a fully defined medium without BPE. Parenthetically, both KGM and KDM have a short shelf life and should not be used after prolonged storage.

For some experiments, the cells were seeded on glass coverslips inserted into a plastic Petri dish and grown in KGM. At the end of the incubation period the medium was removed, the coverslip left in the dish where it was washed with PBS, removed to fix in cold acetone and stained. The coverslip was then inverted on a glass slide over Tris-buffered glycerol and examined with fluorescence microscopy.

Cell Harvesting and Cell Counts

At the times indicated, cultures were washed once with PBS, 1 ml of 0.3% trypsin/ 0.1% EDTA in GNK (150 mM NaCl, 0.04% KCl, 0.1% glucose, 0.084% $NaHCO_3$, pH 7.3) was added, and the plates incubated for 10 min at 37 degrees. The detached cells were transferred to tubes, the plates rinsed x 1 with complete medium to remove the

residual cells and this rinse combined with the 1 ml aliquots already harvested. The cells were diluted with trypan blue and total and viable cell numbers determined using a hemocytometer.

RESULTS

Effect of Culture Medium on rIFN-γ Induction of Class II Antigen Expression by Keratinocytes

Keratinocytes grown in KGM at the high calcium ion concentration (1.8 mM) showed HLA-DR expression by 90% of the cells on day 2 after addition of rIFN-γ (100 U/ml) (Table 1). On this day, significant, albeit less, expression of DP and minimal expression of DQ occurred. In contrast, keratinocytes grown in complete medium showed a different pattern of class II MHC antigen expression in the presence of rIFN-γ (Table 1). As previously reported, HLA-DR expression was virtually maximal by day 4, a time point when DP and DQ expression was still low (5). Thus, on day 4, only small numbers (11%) of cells expressed HLA-DQ. From that time on, expression of HLA-DP increased whereas expression of HLA-DQ stayed very low (Table 1). Cells grown in complete growth medium plus rIFN-γ were morphologically attenuated after 13 days in culture and, therefore, these experiments were not continued beyond this point.

In order to determine the effect of a low calcium ion concentration (0.1 mM) on the expression of class II MHC antigens by

keratinocytes treated with rIFN-γ, similar experiments were repeated using KGM without additional calcium. The results were similar to those found in high calcium KGM. In the low calcium medium, DR expression was high by day 2 and continued at least until day 18. On day 2, HLA-DP also was expressed. By day 10, the mean expression/cell of DP was 327 linear units/cell compared to 492 linear units/ cell for DR and only 49% of these cells expressed DP compared to 76% for DR (Table 1). Significant HLA-DQ expression appeared on day 4, albeit the expression of this class II MHC antigen again was not as strong as DR. Only about 25% of the cells expressed DQ antigen on this day, but this expression increased with time and eventually reached comparable levels with DP by day 10.

To determine whether the total absence of serum proteins or BPE affected the expression of HLA-DR antigen, keratinocytes were grown in KDM without added proteins and 5, 10 and 13 days after the addition of the lymphokine HLA-DR antigen expression was assessed (Table 2). In contrast to cells grown in KGM, the cells grown in KDM expressed very little DR antigen on day 5 of culture. The induction of DR by rIFN-γ in KDM did not reach the values observed on day 2 for KGM until about day 13 of culture. Thus, serum factors appear to play a major role in the induction of DR antigen expression by rIFN-γ.

Effect of Culture Medium on rIFN-γ Induced Growth Inhibition of Keratinocytes

Since keratinocytes without the growth promoting factors found in BPE do not grow rapidly, we predicted that growth inhibition by rIFN-γ in KDM would be less striking. The growth of keratinocytes was in fact influenced less by rIFN-γ in KDM than in any of the other media used. By day 10 of culture, only a small difference in total cell number was seen in control (0.96 x 10^5 cells/plate) vs. rIFN- treated (0.72 x 10^5 cells/plate) cultures. By day 13, however, a three-fold difference in total number of cells/plate between control (1.38 x 10^5 cells/plate) and rIFN-γ treated (0.45 x 10^5 cells/plate) cultures occurred.

Dose of rIFN-γ Required for Induction of Keratinocyte Class II MHC Antigen Expression vs. Growth Inhibition in KGM

In order to determine whether the concentration of rIFN-γ needed to induce DR expression was equivalent to that which resulted in growth inhibition of normal keratinocytes, we performed a dose response curve for the two parameters determining percent of total cells expressing DR as well as viable cell numbers in one and the same Petri dish (Fig.). Recombinant IFN-γ at 3 U/ml, which by day 8 had not significantly inhibited cell proliferation, did induce DR expression. Similarly, 10 U/ml of the lymphokine, which on day 4 had not resulted in a significant decrease in cell number, had induced DR expression on 48% of the cells on this day. Thus, DR

expression was manifested on keratinocytes before inhibition of growth could be documented. These data may explain the existence of skin diseases (e.g., contact dermatitis) in which keratinocytes express DR antigen, yet no atrophy of the epidermis is apparent either clinically or histologically.

Effect of Trypsin on Class II MHC Antigen Expression by Keratinocytes in KGM

To determine whether the delay in HLA-DQ antigen expression and the smaller numbers of cells demonstrating this antigen was due to increased sensitivity of the HLA-DQ antigen to the effects of trypsin treatment, we performed two types of experiments using KGM. For the first, keratinocytes were scraped off the culture plates and then stained for DR, DQ, and DP. As a control, replicate plates were trypsinized and stained in the conventional manner. After scraping, most of the cells were either totally fragmented or dead making quantitation of staining by FACS analysis impossible on these cell preparations. However, on examination using fluorescence microscopy, most of the few, viable keratinocytes that could be visualized were stained with mAb against DR whereas no cells stained for the DP or DQ antigens. To further explore this question, a second type of experiment was performed. Keratinocytes were seeded on cover slips and 4 days after rIFN-γ treatment, the cells were stained for DR, DQ, and DP while still attached. Whereas most cells expressed HLA-DR at this time, only about 50% demonstrated DP and only a very few cells expressed DQ staining and this only very weakly. Thus, the delayed expression of DQ is not an artifact of trypsinization.

The Effect of the Calcium Ion Concentration on Expression of VM-1 and VM-2 Antigens by Keratinocytes Grown in KGM

To determine whether the switch in calcium ions was causing the human keratinocytes to become more differentiated as has been described in the mouse system (8), the cells were labeled with both the mAbs VM-1 and VM-2, antibodies which stain only basal cells of the epidermis in situ (11,12). On day 4, after changing the calcium ion concentration from 0.1 mM to 1.8 mM, virtually every cell expressed the cell surface antigens which bound the mAbs VM-1 and VM-2 in both types of cultures (data not shown). Thus, changing the calcium ion concentration does not change the proliferating phenotype of the attached cells. That the calcium switch does not induce differentiation of human keratinocytes has been reported recently using other markers of differentiation (16).

Effect of Culture Medium on rIFN-γ Induced Class II MHC Antigen Expression by SCL-1 Cells in KGM and Complete Medium

To determine whether SCL-1 cells, which also can be induced to express HLA-DR, showed a similar pattern of expression of the other two class II MHC antigens as did normal keratinocytes, we examined the DP and DQ antigen expression of this cell type grown in complete medium. In this medium, the SCL-1 cells showed little induction of HLA-DP and DQ until about day 13, when low levels of these antigens could be documented (Table 3). Next we compared the induction of class II MHC antigen expression by the SCL-1 cells and normal keratinocytes, both grown in the presence of low calcium KGM. The expression of HLA-DP and DQ by SCL-1 cells was not improved

significantly (Table 4). Thus, SCL-1 cells do not express DP and DQ antigens after rIFN-γ treatment as readily as untransformed keratinocytes, regardless of whether low or high calcium culture conditions are used.

Discussion

Gamma interferon induces keratinocytes to express not only HLA-DR but also HLA-DP and DQ antigens. In all the media tested, the appearance of these class II MHC antigens appears to be time dependent with DR expression occuring first, followed by DP and then DQ. In KGM, with either low or high calcium ion concentrations, DR and DP expression can be detected within 48 hrs of rIFN-γ treatment, whereas the expression of DQ is delayed. Thus, the induction of expression of class II MHC antigens on keratinocytes by rIFN-γ appears to be related to the duration of exposure to the lymphokine as well as serum factors but not to the relative calcium ion concentration. A low level of DQ induction on keratinocytes has been reported previously (6).

The induction of expression of HLA-DP and DQ antigens by rIFN-γ on transformed cells is not as rapid as in normal keratinocytes. Furthermore, the total number of cells expressing DP and DQ is less than in untransformed keratinocytes. Expression of HLA-DR by cells of skin melanomas but not by basal cell carcinomas has been documented (17,18). In one of four squamous cell carcinomas, DR expression by keratinocytes was observed (19). Based on the results in this paper, it would be interesting to determine whether DP and DQ antigens are expressed by squamous cell carcinomas in situ.

That cells grown in KGM expressed DR antigen at an early point in time and on virtually every cell was surprising. All of these cells also expressed the surface antigens recognized by the mAbs VM-1 and VM-2, antigens expressed only by basal cells in situ (11,12). Thus, it is the highly proliferative basal cell which is growing under these conditions of culture and these cells express HLA-DR antigen almost immediately after rIFN-γ exposure. In contact dermatitis, the lower layers of the keratinocytes in the epidermis, presumably the cells in the proliferating

pool, are most intensely stained with mAb against HLA-DR (2). Thus, the in vitro results are consistent with the in situ observations and suggest that the basal cells may be the keratinocyte subpopulation which augments antigen presentation by Langerhans cells in contact dermatitis.

Keratinocytes grown in KDM do not express class II antigens as rapidly as those grown in KGM, which contains BPE, or in complete medium, which contains serum. It is conceivable that in the intact human epidermis, in vivo, the protein composition is not identical to that found in normal human serum. Possibly, the basement membrane at the dermoepidermal junction acts as a type of filter allowing only those proteins and other substances of lower molecular weight to penetrate to the epidermis. Thus, the defined media used for some of these experiments may more accurately reflect the in vivo situation in the epidermis. Alternatively, serum factors may bind rIFN-γ resulting in less active lymphokine being available for the cell surface receptors for this protein when keratinocytes are cultured in serum containing medium.

Keratinocytes express HLA-DR antigen in a wide variety of skin disorders ranging from contact dermatitis which shows no atrophy of the epidermis to the poikilodermatous form of mycosis fungoides where epidermal atrophy can be pronounced. Our findings that short incubations of keratinocytes with low doses of rIFN-γ induce HLA-DR antigen expression but do not result in pronounced reduction in cell numbers, whereas longer incubations with higher concentrations of the lymphokine induce DR as well as DP and DQ expression and reduce cell proliferation, provide further evidence that these three skin disorders may be mediated by IFN-γ.

We have shown recently that rIFN-γ treated keratinocytes are capable of stimulating allogeneic resting T cells in the presence of recombinant IL-2 in vitro (20). Suprisingly, this stimulation was not inhibited by the addition of a monoclonal antibody against HLA-DR antigen. Thus, the stimulation of allogeneic

lymphocytes by rIFN-γ treated keratinocytes appears to be due to expression of an antigen other than DR. The observation that significant numbers of keratinocytes express HLA-DP antigen on day 4 after the addition of rIFN-γ may provide an explanation for rIFN-γ treated keratinocytes' capacity to stimulate foreign T lymphocytes.

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Figure Legend

Keratinocytes grown in KGM were treated with various concentrations of rIFN-γ. On days 2, 4 and 8 the cells were harvested and the number of viable cells/plate as well as the percent of these cells expressing HLA-DR determined. For the cell counts the SEM was less than 25% except for the control on day 8, where the SEM was 30%. The Table accompanying the Figure indicates the per cent of total cells which express HLA-DR for each concentration of rIFN-γ and for each day of culture.

Table 1

Expression of Class II Antigens by rIFN-γ Treated Normal Keratinocytes as a Function of the Medium Used.[a]

Days after rIFN-γ Treatment	KGM with low Ca(0.1 mM)			KGM with high Ca (1.8 mM)			DMEM plus serum		
	DR	DP	DQ	DR	DP	DQ	DR	DP	DQ
2	81[b]	36	4	90	44	10	N.T.[c]	N.T.	N.T.
4	86	71	25	75	84	21	40	18	11
6	69	77	29	65	52	18	N.T.	41	0
7	N.T.						45	24	0
8	91	77	26	72	50	14	51	17	1
10	76 (492)	49 (327)	44 (89)	N.T.	N.T.	N.T.	42	19	6
12	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	30	34	14
13	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	67	34	5
14	78 (530)	83 (330)	74 (246)	N.T.	N.T.	N.T.			
18	64 (376)	65 (339)	63 (158)	N.T.	N.T.	N.T.			

[a] Composite of several experiments.

[b] Number of positive cells expressed as % of total; mean fluorescence/cell (in linear units) is given in parentheses. The isotype controls have been subtracted for both values.

[c] N.T. = not tested. In prior experiments, DR expression was low on day 2

TABLE 2

Expression of HLA-DR by Keratinocytes in

KDM plus rIFN-γ (100 U/ml)

Days in Culture	Number of Positive Cells (% of total)[a]	Mean Fluorescence per Cell[a]
5	10	11
10	31	211
13	76	663

[a] The isotype control has been subtracted.

TABLE 3

Class II Antigen Expression by rIFN-γ Treated SCL-1 Cells in Complete Medium

	Class II Antigen Staining[a]		
Day	DR	DP	DQ
2	50[a]	1	1
4	69	1	3
6	N.T.[b]	6	7
7	94	5	5
10	74	2	5
13	81	12	8
14	77	30	22

[a] Number of positive cells (% of total); the value for the isotype controls has been subtracted.

[b] N.T. = not tested.

TABLE 4

Expression of Class II MHC Antigens by rIFN-γ Treated SCL-1 Cells and Normal Keratinocytes in Low Calcium Medium (KGM)

Cells	Days after rINF-γ	Class II Antigen Expression[a] DR	DP	DQ
SCL-1	2	74	2	2
	4	56	1	3
	6	71	1	3
	8	75	1	0
	11	82	6	2
	14	88	11	25
Normal Keratinocytes	2	N.T.[b]	N.T.	N.T.
	4	86	71	25
	6	69	77	29
	8	91	77	26
	10	76	49	44
	14	78	83	74
	18	64	65	63

[a] Number of positive cells (% of total). The value for the isotype controls has been subtracted.

[b] N.T. = not tested.

TABLE for Figure

Time after rIFN-γ Treatment (days)	Concentration of rIFN-γ (U/ml)	Number of Cells Expressing HLA-DR (%)[a]
2	3	3
	10	48
	30	77
	100	87
4	3	1
	10	48
	30	89
	100	96
8	3	13
	10	75
	30	93
	100	91

[a] Isotype control has been subtracted.